AWARD NUMBER: W81XWH-16-1-0692

TITLE: Selective AAK1 and GAK Inhibitors for Combating Dengue and Other Emerging Viral Infections

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RECIPIENT: Geneva Foundation
Tacoma, WA 98402

REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Annual	15 Sep 2016 - 14 Sep 2017

4. TITLE AND SUBTITLE
Selective AAK1 and GAK Inhibitors for Combating Dengue and Other Emerging Viral Infections

5a. CONTRACT NUMBER

5b. GRANT NUMBER
15W81XWH -16-1-0692

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Geneva Foundation
Tacoma, WA 98375

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
USAMRMC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We discovered an Achilles' heel of unrelated viruses: a requirement for AP2-associated protein kinase 1 (AAK1) and cyclin G-associated kinase (GAK), host kinases that regulate clathrin adaptor proteins-mediated pathways. Our data point to AAK1 and GAK as "master regulators" of viral infection and attractive targets for broad-spectrum antivirals. We discovered that approved anticancer drugs that target these kinases; sunitinib and erlotinib, potently inhibit replication of multiple viruses *in vitro* and reduce mortality in mice infected with DENV and EBOV. This approach is now being advanced to the clinic for both of these indications. Nevertheless, while sunitinib and erlotinib are quite potent inhibitors of AAK1 or GAK, respectively, they are not selective and are therefore associated with toxicity resulting from inhibition of other host cell kinases. The goals of this proposal are to: optimize novel, chemically distinct, selective lead AAK1 and GAK inhibitors targeting validated virus-host interactions and already demonstrating great promise against DENV, and advance their development to a near-IND stage. This approach would also protect against biothreat agents from eight viral families, including EBOV and CHIKV.

15. SUBJECT TERMS
None listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

USAMRIID will determine antiviral activity of the optimized compounds alone and in combinations, and demonstrate efficacy with no toxicity in best available murine models of emerging viruses. The overall goal is to develop broad-spectrum antiviral drugs with a high genetic barrier to resistance by targeting host proteins that are critical to the life cycle of multiple viruses.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Broad-spectrum, anti-viral, filovirus, Ebola virus, Marburg virus, alphavirus, chikungunya virus, mouse model

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Optimize lead inhibitors of AAK1 and/or GAK and maximize their therapeutic index.

Major Task 1: Optimize the metabolic stability and pharmacokinetic (PK) properties of the current leads. Milestone achieved: At least one AAK1 and/or one GAK inhibitor that is metabolically stable and maintains its kinase activity and selectivity are identified (month 14).

Major Task 2: Determine antiviral activity of the optimized compounds alone and in combinations. Milestones achieved: i. In vitro efficacy against at least 1 DENV serotype and an unrelated virus demonstrated (with EC_{50}<5μM and CC_{50}>10xEC_{50}) (month 15); ii. Lack of antagonism combined with lack of cumulative toxicity with at least 1 class of anti-DENV drug demonstrated (month 24).

Specific Aim 2: Determine the in vivo metabolism, activity, and relative barrier to resistance of the AAK1 and GAK inhibitors.

Major Task 3: Determine PK and demonstrate efficacy with no toxicity in best available murine models of emerging viruses. Milestones achieved: i. AAK1 and GAK inhibitors with lengthened *in vivo* half-life identified (month 20); ii. Decreased mortality of animals (month 24); iii. >1 log reduction of viral titer in blood or tissues with no drug related morbidity or mortality (month 24).

Major Task 4: Determine the potential for emergence of drug resistance compared to direct acting antiviral (DAAs). Milestones achieved: i. Delayed time to and low level of resistance demonstrated *in vitro* (month 24); ii. Delayed time to and low level of resistance shown *in vivo* (month 36).

Specific Aim 3: Select AAK1 and GAK inhibitors as pre-IND candidates.

Major Task 5: Conduct *in vitro* ADME-toxicity studies and initial preclinical animal safety testing on the optimized leads and their major metabolites. Milestones achieved: i. Key *in vitro* ADME-Tox parameters determined (month 30); ii. Maximum tolerated dose (MTD) defined (month 36); iii. Initial preclinical *in vivo* toxicity assessment demonstrating no red flags (month 36).

Specific Aim 4: Validate the mechanism of action (MOA) of selective AAK1 and GAK inhibitors in DENV infection.

Major Task 6: Validate the molecular targets underlying the antiviral effect of AAK1 and GAK inhibitors and examine these compounds' binding to their predicted antiviral target and its modulation. Milestones achieved: i. >2 fold increase in EC_{50}s for the drugs' antiviral effect following overexpression of AAK1 and GAK in infected cells and infected animals ("rescue experiments") (month 30 in vitro); ii. >2 fold decrease in AP2 phosphorylation in PBMCs derived from DENV infected mice on treatment (month 36 in vivo).

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

This year, USAMRIID has tested nine candidate inhibitors. Inhibitors were evaluated for their impact on *in vitro* viability prior to evaluation of inhibitory capacity against Ebola virus (EBOV), Marburg virus (MARV) and/or chikungunya virus (CHIKV). For viability assessment, compounds were applied to cells at a range of doses, and a commercially available kit was used to measure ATP content as a marker of viability. Only compounds/doses that did not decrease viability below 70% that of vehicle were moved forward to live virus evaluation. Briefly, cells were pretreated with inhibitor, incubated with virus for one hour without inhibitor present, washed, and then media containing inhibitor was added to cells. At indicated time points, plates were fixed and removed from the suite, and virus infection was measured by immunofluorescence using a benchtop high content imaging system.

A summary of all compounds testing to date is found in Table 1 (page 17). The following compounds were evaluated against EBOV *in vitro*: azaindole, 2787, 2789, 2892, 2983, 7-z-oxozeaenol and 2608. All of these compounds demonstrated activity at some level against EBOV (Figures 1-2 and data not shown, page 14-15). The same compounds, with the exception of 2983, were tested *in vitro* against MARV; all but 2608 showed efficacy (Figures 1-2 and data not shown, page 14-15). Finally, azaindole, 2787, 2608, 1966 and 1967 were tested *in vitro* against CHIKV; all but 1967 demonstrated efficacy against CHIKV (Figure 3 and data not shown, page 16). These data will be used to prioritize optimization of compounds for animal studies going forward.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Two members of the Dye lab had the opportunity for professional development as a result of this project. Results from USAMRIID were presented at two conferences during the first year of this project. Dr. Brannan presented a poster at the Keystone Hemorrhagic Fever Viruses meeting in Santa Fe, New Mexico in December of 2016. Ms. Laura Prugar presented a poster at the 9th Annual Symposium on Filoviruses in Marburg, Germany, in September 2017.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Results were presented at two scientific conferences (see professional development).

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During the next year, we will confirm lead hits and test modifications of lead candidates *in vitro*, prior to evaluating the efficacy of lead candidates in rodent models of infection.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Compounds tested for this project were used to aid in the development of an immunofluorescence assay for evaluating chikungunya virus infection. This will aid in screening for chikungunya virus antivirals.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Not applicable.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Brannan JM, et al. Inhibition of host kinases AAK1 and GAK during Ebola virus infection. Keystone Symposium on Hemorrhagic Fever Viruses, December 4-8, 2016; Santa Fe, NM; Poster. Prugar, LI, et al., AAK1 and GAK inhibitors demonstrate activity against multiple RNA viruses 9th Symposium on Filoviruses, September 13-16, 2017, Marburg, Germany; Poster.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

  Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name: Dr. John Dye
Project Role: Principal Investigator
Nearest person month worked: .06 calendar months
Contribution to Project: Supervisory

Name: Dr. Jennifer Brannan
Project Role: Co-Investigator
Nearest person month worked: 2.73 calendar months
Contribution to Project: Design, analysis and oversight of filovirus and alphavirus *in vitro* evaluations of inhibitors

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report." If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Title:	Assessing and Characterizing the Protective Efficacy of Convalescent of Human Plasma from Ebola Virus Survivors as a Treatment for Patients with Acute Ebola Virus Disease
POP:	10/01/2014-09/30/2017
Change in Support:	Previously active, but now closed.

Title:	Structure-guided Redesign of Monoclonal Antibodies Targeting Conserved Filovirus Epitopes
Effort:	5% Dye; 5% Brannan
Agency:	NIAID
POP:	07/01/2016-06/30/2018
Funding:	$284,365
Change in Support:	Previously pending, but now active.
Title:	Evolution of Anti-filovirus B Cell Responses and Mechanisms of Protection
Effort:	5% Dye; 5% Brannan
Agency:	NIAID
POP:	04/01/2017-03/31/2022
Funding:	$754,197
Change in Support:	Previously pending, but now active.
Title:	Engineered, Multispecific Antibodies as Broad Anti-filovirus Therapeutics
Effort:	5% Dye
Agency:	NIAID
POP:	06/01/2017-05/30/2018
Funding:	$115,501
Change in Support:	Previously pending, but now active.
Title:	"A Trojan Horse" Bispecific Antibody Strategy for Broad Filovirus Therapeutics
Effort:	5% Dye
Agency:	NIAID
POP:	07/01/2017-06/30/2018
Funding:	$117,041
Change in Support:	New project
Title:	Broadly Protective Bispecific Antibodies for Treatment of Ebola Virus Disease
Effort:	5% Dye
Agency:	NIAID
POP:	07/01/2017-06/30/2022
Funding:	$482,206
Change in Support:	New project
Title:	Dissecting the Receptor-mediated Infection Mechanisms of Hantavirus
Effort:	5% Dye
Agency:	NIAID
POP:	07/01/2017-06/30/2022
Funding:	$96,281
Change in Support:	New project

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Organization Name: Geneva Foundation
Location of Organization: Tacoma, WA

Organization Name: Stanford University
Location of Organization: Stanford, CA
Partner's contribution to project: Collaboration

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

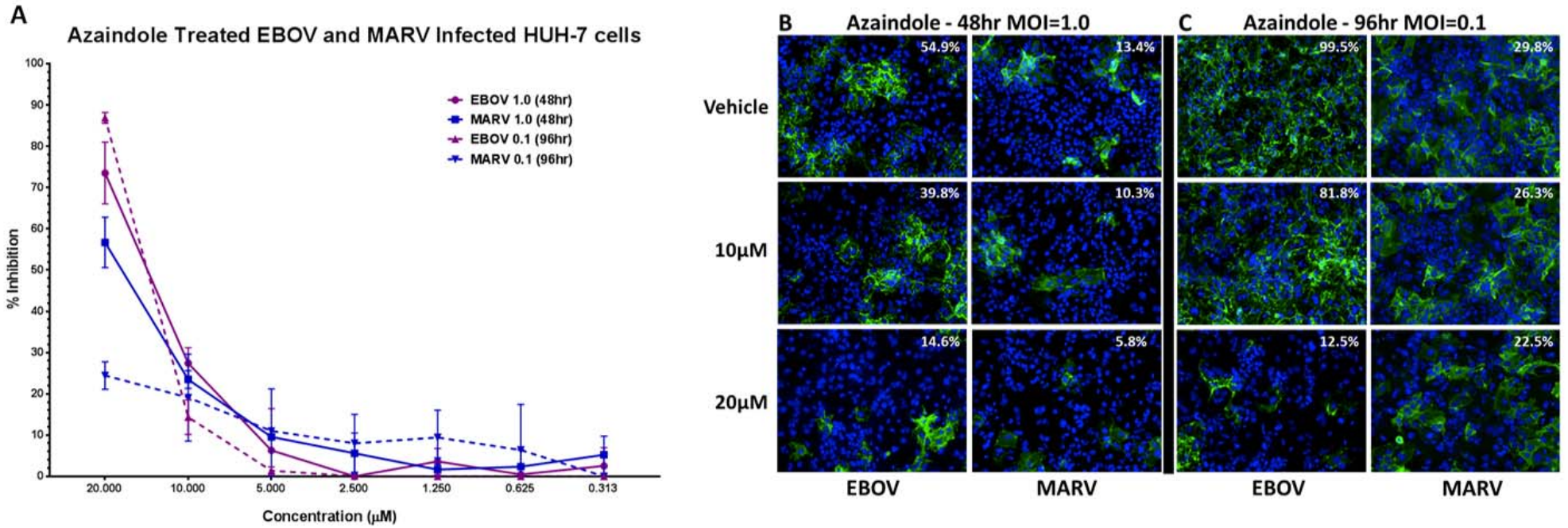

Figure 1: Azaindole inhibits EBOV and MARV infection in HUH-7 cells. Following a pre-treatment for 1 hour with kinase inhibitor Azaindole, HUH-7 cells were infected for 1 hour with EBOV or MARV at MOI=1.0 and 0.1, in the presence of inhibitor. Cells were washed with PBS prior to re-adding media containing Azaindole at several dilutions or Vehicle control, and incubated at 37°C for either 48hours (MOI=1.0) or 96 hours (MOI=0.1). Supernatants were collected (20μM or 10μM), pooled, and clarified for later analysis by plaque assay. Cells were fixed, then stained with virus specific monoclonal antibodies. Cells were imaged using a PerkinElmer *Operetta*® High Content Imaging System and the percent of infected cells was determined using PerkinElmer Harmonia software. Data is expressed as percent of vehicle control treated cells (A). Representative IFA images are shown for both MOIs and time points (B and C).

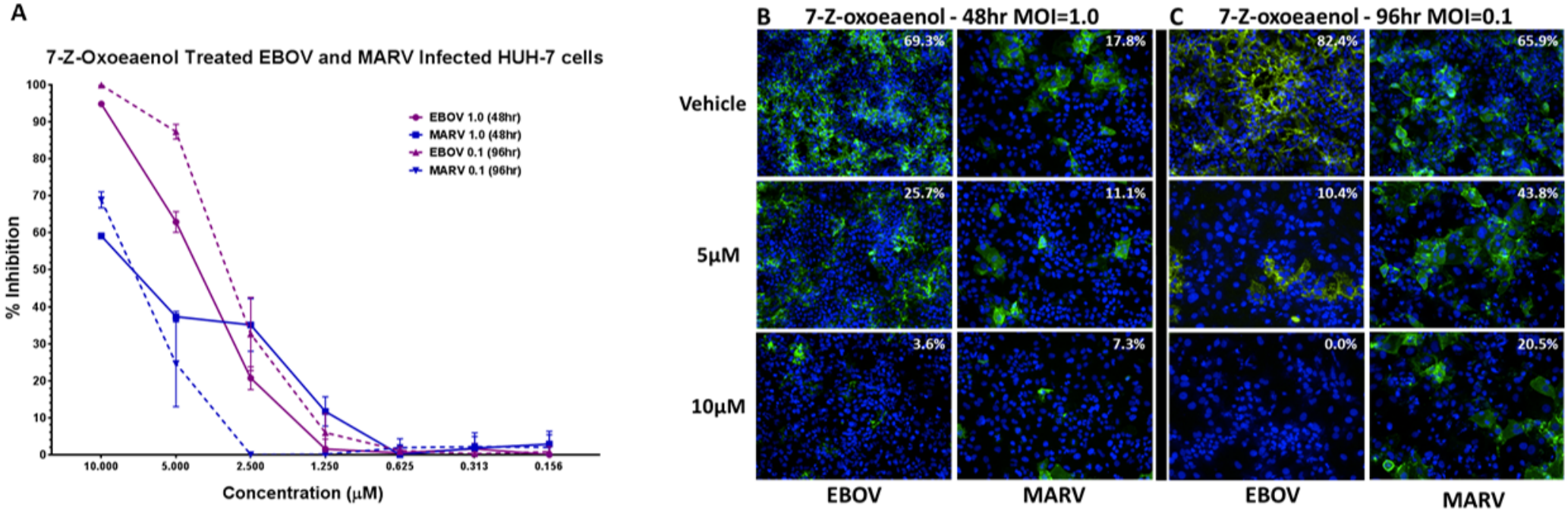

Figure 2: 7-Z-oxoeaenol inhibits EBOV and MARV infection in HUH-7 cells. Following a pre-treatment for 1 hour with kinase inhibitor 7-Z-oxoeaenol, HUH-7 cells were infected for 1 hour with EBOV or MARV at MOI=1.0 and 0.1, in the presence of inhibitor. Cells were washed, media containing 7-Z-oxoeaenol at or vehicle control was added, and cells were incubated at 37°C for either 48hours (MOI=1.0) or 96 hours (MOI=0.1). Following incubation, cells were fixed, and stained with virus specific monoclonal antibodies. Cells were imaged using a PerkinElmer *Operetta*® High Content Imaging System and the percent of infected cells was determined using PerkinElmer Harmony software. Data is expressed as percent of vehicle control treated cells (A). Representative IFA images are shown for both MOIs and time points (B and C).

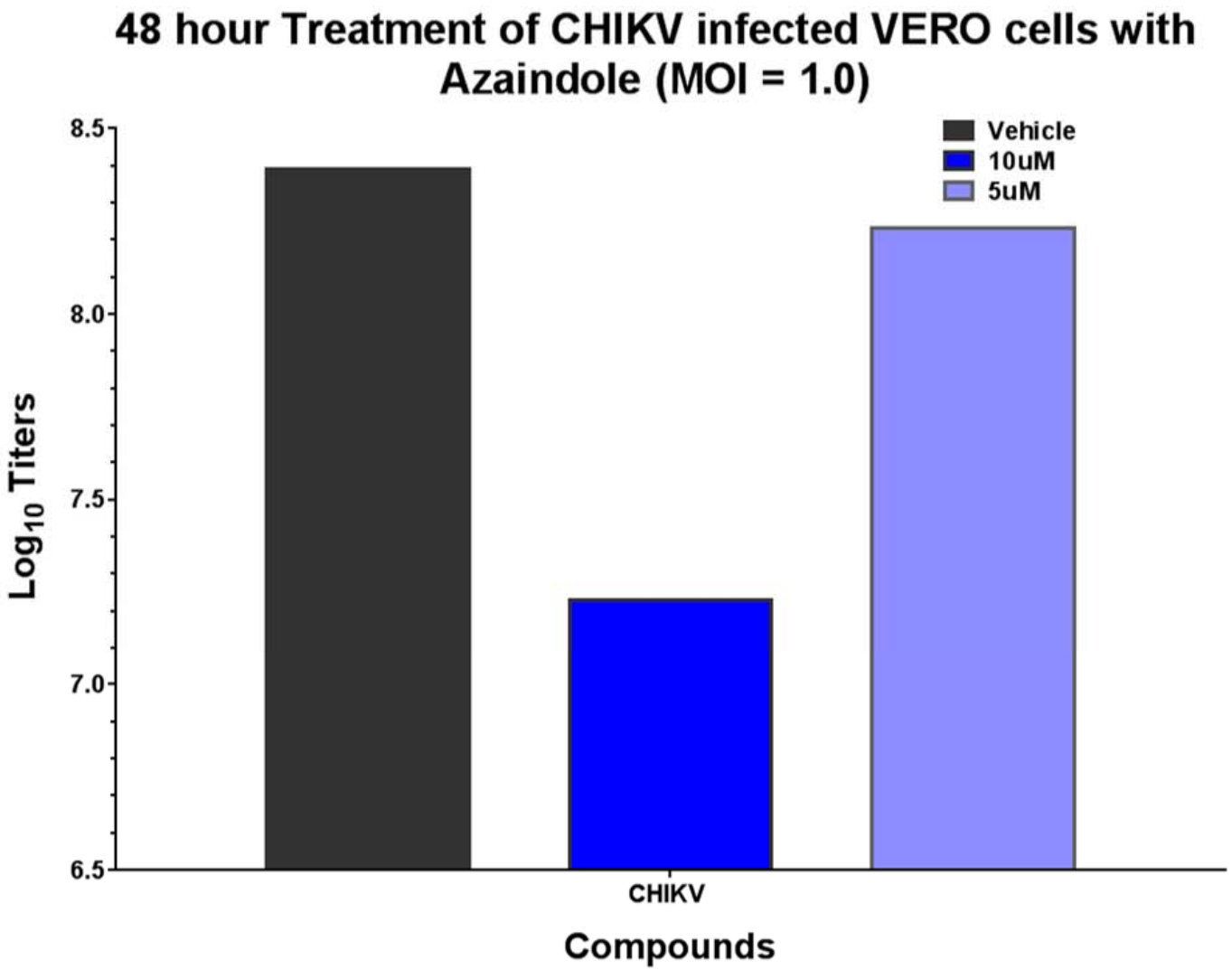

Figure 3. Reduction of viral titers following Azaindole treatment of CHIKV infected VERO cells. Following a pre-treatment for 48 hours with kinase inhibitor Azaindole, Vero cells were infected for 1 hour with CHIKV at MOI=1.0. Cells were washed with PBS prior to re-adding media containing Azaindole (10µM or 5µM) or Vehicle control, and incubated at 37°C for 48hours (MOI=1.0), then supernatants were collected and clarified, and plaque assays were performed to determine viral titers. Data is expressed as Log_{10} transformed titers in plaque forming units/ml.

Table 1: Summary of *in vitro* evaluation of kinase inhibitor panel

Compound	*In vitro* inhibition observed		
	EBOV	MARV	CHIKV
azaindole	yes	yes	yes
2787	yes	yes	yes
2789	yes	yes	nd
2892	yes	yes	nd
2983	yes	nd	nd
7-z-oxozeaenol	yes	yes	nd
2608	yes	no	yes
1966	nd	nd	yes
1967	nd	nd	no